RAPID COMMUNICATION

Presence and Expression of Human Papillomavirus Sequences in Human Cervical Carcinoma Cell Lines

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A series of human carcinoma cell lines was examined for human papillomavirus (HPV) DNA sequences with the use of HPV-6, HPV-11, HPV-16, and HPV-18 DNA probes. Six of eight cell lines derived from human cervical carcinomas were shown to contain integrated HPV DNA sequences. In five of these six lines, HPV-specific polyadenylated RNA species could also be identified. The expression of HPV sequences was detected in three lines with a HPV-18 DNA probe and in two lines with a HPV-16 DNA probe. Of the two lines which contained HPV-16 specific RNA, one contained HPV DNA sequences which hybridized only to an HPV-16 probe, and the other contained HPV DNA sequences which hybridized to both HPV-16 and HPV-18 DNA probes. Six cell lines established from human squamous-cell carcinomas of the bladder, pharynx, lung, esophagus, and vulva were negative for HPV-6, HPV-11, HPV-16, and HPV-18 DNA sequences under stringent hybridization conditions. (Am J Pathol 1985, 119:361–366)

THE PAPILLOMAVIRUSES are a group of small DNA viruses associated with benign squamous epithelial tumors in higher vertebrates. In man, at least 27 distinct human types (HPVs) have now been identified, and many of these have been associated with specific clinical lesions. Four of these, HPV-6, HPV-11, HPV-16, and HPV-18, have been associated with human genital tract lesions. HPV-6 DNA, which was cloned from a condyloma accuminata,[1] and HPV-11 DNA, which was cloned from juvenile laryngeal papilloma,[2] have been found to be associated with a high percentage of benign genital warts and cervical flat warts or dysplasias.[3] HPV-16 DNA was cloned directly from a cervical carcinoma biopsy[4] and has been demonstrated in a high percentage of cervical, vulvar, and penile cancer biopsies. In addition, the DNA has been found in biopsy specimens of bowenoid papulosis,[5] Bowen's disease,[5] and some cervical dysplasias characterized by aneuploidy.[6] HPV-18 DNA was also cloned directly from a cervical cancer biopsy specimen, and it has been found in 11 of 49 cervical carcinoma biopsies, in one penile carcinoma biopsy, and in three cell lines derived from cervical carcinoma.[7] In general, HPV-6 and HPV-11 DNAs have been found associated with benign lesions of the genital tract, whereas HPV-16 and HPV-18 DNAs have been found associated with premalignant and malignant lesions. We have found that HPV-16 and HPV-18 DNAs are also present and are transcribed in most cell lines established from human cervical carcinomas.

Materials and Methods

Cell Culture

The following human carcinoma cell lines were obtained from the American Type Culture Collection: C-33A, HT-3, ME 180, MS751, SiHa, C-4I and C-4II, HeLa, CaSki, Scaber, FaDu, and SK-MES-1. The A431 line was obtained from Kurt Stromberg (NCI, NIH). Human esophageal carcinoma lines TE-1 and TE-2 were obtained from Dr. Banks-Schlegel (NCI, NIH). All lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO).

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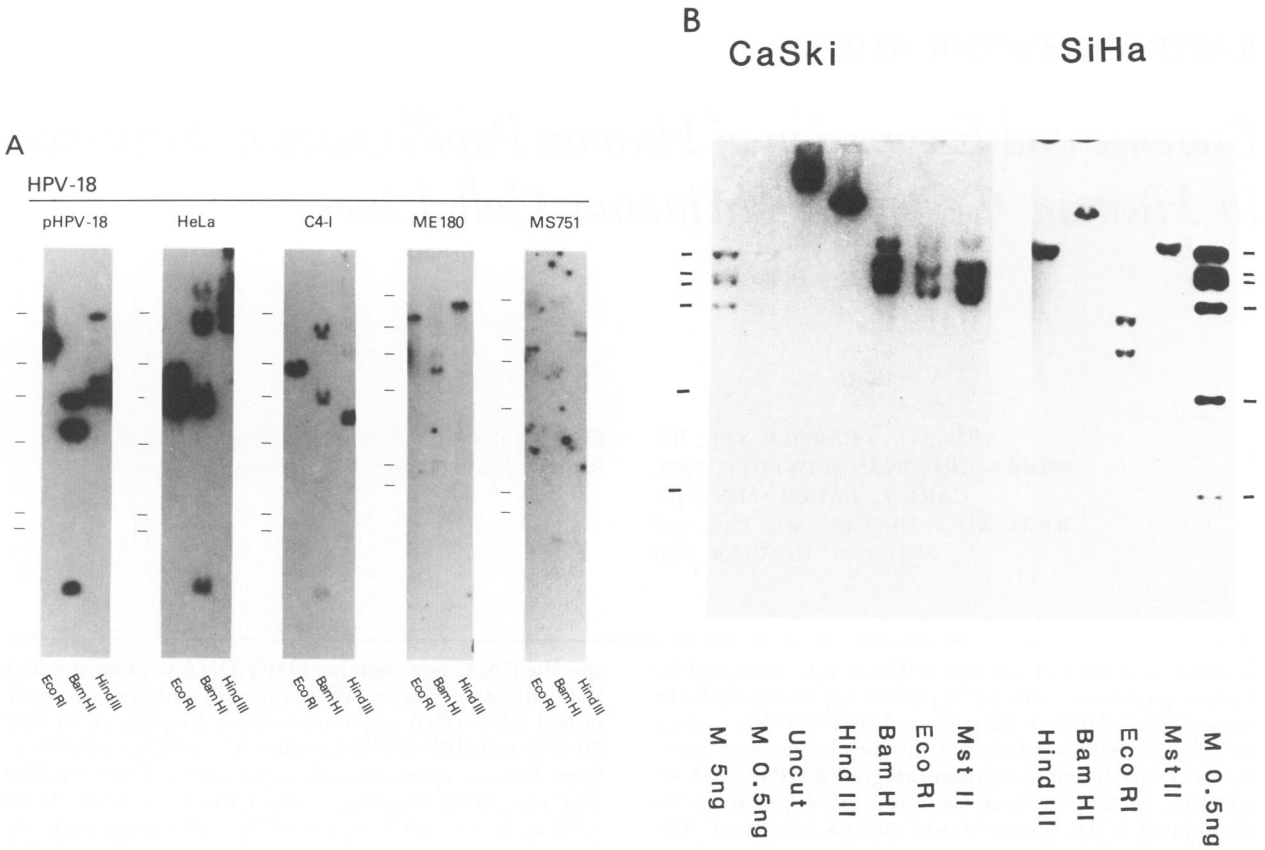

Figure 1—Hybridization of DNA from human cervical cell lines with HPV-16 or HPV-18 DNA probes. **A**—Analysis of DNA from the HeLa, C-4 I, ME-180, and MS-751 cell lines for the presence of HPV-18 DNA. Cellular DNA was isolated from each of the indicated cell lines and then electrophoretically separated and blotted onto nitrocellulose as described in Materials and Methods. Purified HPV-18 insert DNA was nick-translated[29] and used for hybridization. The left lanes contain 100 pg of cloned HPV-18 DNA (10 virus genomes per cell genome equivalent) cleaved with the indicated restriction-endonuclease and mixed with 10 μg salmon sperm DNA. Molecular weight markers indicated on the left of each blot are *Hind*III fragments of lambda DNA of the following sizes: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. **B**—Analysis of DNA from the CaSki and SiHa cell lines for the presence of HPV-16 DNA. Cellular DNA (10 μg) was electrophoretically separated, blotted, and hybridized with HPV-16 (*Bam*HI linear) DNA as described in Materials and Methods. The DNA size markers consist of *Bam*HI linear cloned HPV-16 DNA cut with various restriction endonucleases to yield fragments of the following sizes: 7.9, 6.6, 6.1, 5.0, 2.9, and 1.8 kb. Each marker lane (labeled M) contained 5 or 0.5 ng total HPV-16 DNA. The upper band (7.9 kb) in each marker lane is equivalent to 125 virus genome equivalents and 12.5 virus genome equivalents per cell genome equivalent for the 5 ng and 0.5 ng lanes, respectively.

DNA/DNA Hybridization

Cellular DNAs (10 μg) were digested with indicated restriction-endonucleases, electrophoresed through a 1% agarose gel, and transferred onto a nitrocellulose filter as previously described.[8] HPV DNA was purified from the pBR322 or pML2d plasmid in which it was cloned and was nick-translated to a specific activity of 1.0×10^8 cpm/μg DNA. Hybridization and subsequent washings were performed under stringent conditions of approximately $T_m - 25$ C.[9] The blots were exposed for 1–10 days at −70 C before development.

RNA/DNA Hybridization

Total cellular RNA was obtained by lysis of cells in 4 M guanidinium thiocyanate sedimentation through a CsCl cushion and ethanol precipitation.[10] For slot blot analysis, 10 μg of total cell RNA from the indicated cell line was dissolved in 0.2 ml of 10 × SSC and bound to each slot by slow filtration onto a nitrocellulose membrane.[11]

The poly(A)$^+$ RNA was obtained by two cycles of oligo (dT)-cellulose chromatography.[12] Poly(A)$^+$ RNA (1 μg) was fractionated on 1% formaldehyde–agarose gels and transferred to nitrocellulose filters. Hybridization was done with a HPV-16 probe which was purified from the vector DNA prior to ^{32}P-labeling by nick translation to a specific activity of 10^8 cpm/μg. Hybridization was performed in 50% formamide, 5× SSC for 24 hours at 42 C. Filters were washed in 0.1× SSC 0.1% SDS at 60 C and exposed to x-ray films for 1–2 days with intensifying screens.

Filters (from the slot blot analysis and gel electropho-

Table 1—Presence and Expression of Human Papillomaviruses in Human Cancer

Cell Line	Cancer Type	HPV DNA				HPV RNA*		Reference
		6	11	16	18	16	18	
C-33A	Cervix	–	–	–	–	–	ND	14
HT-3	Cervix	–	–	–	–	–	ND	15
ME 180	Cervix	–	–	–	+	–	–	16
MS751	Cervix	–	–	–	+	–	+	16
SiHa	Cervix	–	–	+	–	+	–	17
C-4 I and C-4 II†	Cervix	–	–	–	+	–	+	18
HeLa	Cervix	–	–	–	+	–	+	19
CaSki	Cervix	–	–	+	+‡	+	–	20
Scaber	Bladder	–	–	–	–	–	–	21
FaDu	Pharynx	–	–	–	–	ND	ND	22
SK-MES-1	Lung	–	–	–	–	ND	ND	15
A431	Vulva	–	–	–	–	ND	ND	23
TE-1	Esophagus	–	–	–	–	ND	ND	24
TE-2	Esophagus	–	–	–	–	ND	ND	24
Normal spleen	–	–	–	–	–	ND	ND	

* ND, not done.
† These two morphologically distinct cell lines were isolated from the same tumor and contain an identical pattern of integrated HPV-18 DNA sequences.
‡ Hybridization of HPV-18 DNA was detected below $T_m - 25$ C but not under more stringent annealing conditions.

resis) were baked and prehybridized and hybridized at 42 C for 4 hours with a mixture of 5× SSC, 5× Denhardt's solution,[13] 50 mM sodium phosphate buffer (pH 6.5), 50% deionized formamide, sheared denatured salmon sperm DNA (200 μg/ml), and heat-denatured ^{32}P-labeled HPV-18 or HPV-16 DNA (10^8 cpm/μg). The filters were subsequently washed three times with 2× SSC, 0.1% SDS at room temperature for 15 minutes and then washed three times for 30 minutes each with 0.1× SSC and 0.1% SDS at 60 C.[11]

Results

Detection of HPV DNA

Fourteen human carcinoma cell lines were evaluated for the presence of HPV sequences using HPV-6, 11, 16, and 18 DNAs as probes. Of eight cervical carcinoma cell lines examined, six were found to contain HPV DNA sequences using HPV-16 and HPV-18 DNA probes under stringent hybridization conditions. Four of the eight cell lines contained DNA sequences which hybridized with an HPV-18 DNA probe under stringent conditions. The results on these four cell lines are presented in Figure 1A. Two of these cell lines, HeLa and C4-I, have previously been shown to contain HPV-18 DNA sequences.[7] All of the viral DNA sequences are apparently integrated, because no extrachromosomal plasmid viral DNA sequences could be detected following cleavage with a presumed "no cut" restriction endonuclease for the viral DNA (*Xho* I) or after mild shearing of the cellular DNA. The extent of the hybridization with the HPV-18 DNA probe varied among the cell lines. Sequences were readily detected in HeLa cells, which have been previously shown to contain approximately 10 copies of the viral genome integrated in an apparent head-to-tail manner, and in C4-I cells, which have been reported to contain an estimated one copy per diploid genome.[7] Hybridization to specific DNA sequences could also be detected in the ME 180 and MS751 cervical carcinoma cell lines but only after long exposure to the x-ray film, and the intensity of hybridization was judged to be equal to or less than one copy per cell (Figure 1A). The variations among the restriction endonuclease cleavage patterns of the tumor cell line DNAs could be due to the site of integration of the viral DNA, rearrangements and/or deletions associated with the integrative event, or HPV DNA strain variability. Further analyses of the structure of these integrated viral DNAs are in progress.

No specific hybridization was detected with an HPV-18 DNA probe with DNA from the SiHa, C-33A, or HT-3 cervical carcinoma cell lines. HPV-specific sequences were not detected in human spleen DNA or DNA extracted from several human squamous-cell carcinoma cell lines examined: Scaber (bladder), FaDu (pharynx), SK-MES-1 (lung), A431 (vulva), TE-1 (esophagus), and TE-2 (esophagus) (Table 1).

Two cell lines (CaSki and SiHa) contain DNA sequences which hybridized intensely with the HVP-16 DNA probe (Figure 1B). The CaSki line contains greater than 500 copies, and SiHa is estimated to contain approximately ten copies of the DNA hybridizing to the HPV-16 probe. The DNA sequences are apparently integrated, because no monomer circular forms of DNA (8 kb or smaller) were detected in uncleaved samples of cellular DNA. The simple pattern of hybridization indicates that the DNA is probably integrated in a head-

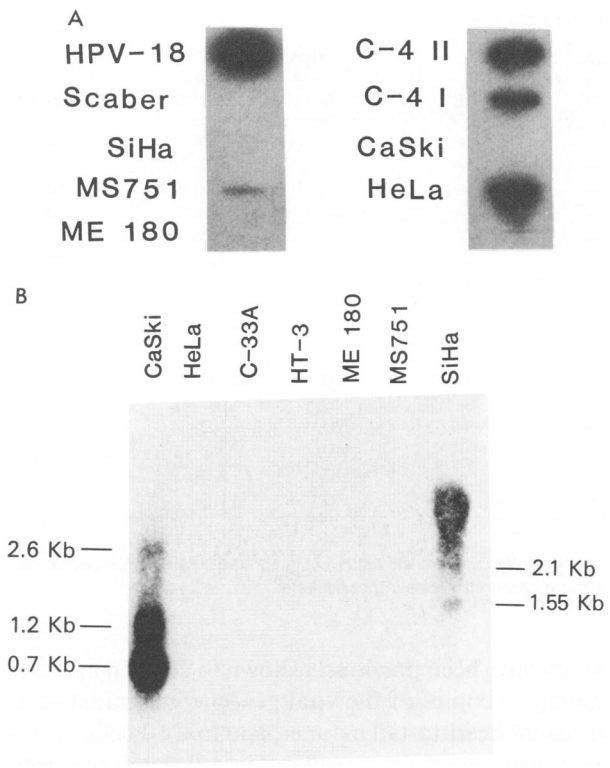

Figure 2—Hybridization analysis of RNA from cervical carcinoma lines for HPV-16 and HPV-18 specific sequences. **A**—Slot blot analysis of total RNA from human carcinoma cell lines. Total RNA (10 μg) from each of the indicated cell lines was blotted and hybridized to HPV-18 DNA as described in Materials and Methods. **B**—Northern blot analysis of poly(A)$^+$ RNA from human cervical cancer cell lines. One microgram poly(A)$^+$ RNA from the indicated cell lines was electrophoretically separated, blotted, and hybridized with purified HPV-16 DNA as described in Materials and Methods.

to-tail tandem array in each cell line. Replica blots of the DNA from the CaSki line indicated that certain of the HPV sequences which hybridized to the HPV-16 probe apparently also hybridize with the HPV-18 probe under slightly less stringent conditions, which suggests that the HPV DNA sequences integrated in the CaSki cell line are related to both HPV-16 and HPV-18 DNAs. Under these annealing conditions, no cross-hybridization could be detected between the cloned HPV-16 and HPV-18 DNAs. Thus, we conclude that the HPV sequences integrated in the CaSki cell lines may represent a new HPV related closely to HPV-16 and more distantly to HPV-18. In the C-33A and HT-3 cell lines, no specific hybridization could be detected under stringent hybridization conditions at $T_m - 25$ C after long exposure with the use of either an HPV-16 DNA or an HPV-18 DNA probe. No HPV-16 DNA sequences were detected in the remaining six cervical carcinoma lines, and no hybridization was detected in the normal spleen DNA or in the DNA from the bladder, pharynx, lung, esophagus, and vulvar squamous-cell carcinoma lines. In addition, no specific hybridization was detected for any of these cellular DNAs with either an HPV-6 or HPV-11 DNA probe. Table 1 presents a summary of these hybridization results.

Detection of HPV RNA

We next examined whether viral specific transcription could be detected in the cell lines containing HPV DNA sequences. Total cellular RNA was isolated from the cervical carcinoma lines positive for HPV DNA sequences and from the bladder carcinoma line (Scaber) as a control and analyzed by slot-blot hydridization using an HPV-18 specific radiolabeled probe. As shown in Figure 2A, RNA which hybridized to an HPV-18 probe could be detected in three of six independent lines examined. The C-4I and C-4II lines are two morphologically distinguishable subclones of the same cervical carcinoma lines. No HPV RNA could be detected in the ME 180 cell line, which does contain HPV-18 DNA. The CaSki and SiHa lines, which contain HPV-16 DNA, are negative for HPV-18 RNA sequences, as is the control bladder carcinoma cell line, Scaber. The polyadenylated HPV RNA species present in HeLa cells and in C4-I cells were further analyzed by fractionation in formaldehyde gels and found to be similar in size to those reported.[25] The polyadenylated HPV RNA species detected in the MS751 cells were approximately 1.5 kb in size (data not shown).

We also examined these cell lines for HPV-16 polyadenylated RNA. Each of the two lines which contained HPV sequences which hybridized to an HPV-16 DNA probe contain HPV-16 specific RNAs (Figure 2B). In the CaSki cell line the HPV-16 specific RNAs are abundant, and three size classes measuring 0.7, 1.2, and 2.6 kb are noted. In the SiHa cell line, distinct species measuring 1.55 kb and 2.1 kb in size were detected as well as a more heterogeneous class of RNAs, ranging up to 6.5 kb in size.

Discussion

The finding that six of eight human cervical carcinoma cell lines examined in this study contain integrated HPV sequences (detected either with an HPV-16 probe or with an HPV-18 DNA probe) further strengthens the association of the HPVs with human cervical carcinoma. These findings indicate that HPV-16–related sequences can be present in human cervical carcinoma cell lines. Although HPV-16 DNA has been detected in biopsies of cervical tumors,[25] this is the first report of its presence in a carcinoma cell line. HPV-18 DNA had been reported in three of three cervical carcinoma cell lines that had previously been examined, and it had been

argued that the presence of HPV-18 DNA might promote establishment of lines in tissue culture.[7] Thus, any requirement for HPV sequences for the establishment of a cell line would not appear to be strictly limited to HPV-18.

HPV-16 and HPV-18 may only be two members of a larger related group of HPVs which may be associated with genital tract neoplasias. As discussed in the Results section, the presence of HPV viral sequences in the CaSki cell line which can be detected with either HPV-16 or HPV-18 probes under different hybridization conditions indicates that this cell line may contain a new HPV genome partially related to the prototype HPV-16 and HPV-18 genomes already described. Although we failed to detect HPV sequences in the C-33A or HT-3 cell lines using HPV-6, HPV-11, HPV-16, and HPV-18 DNA probes under stringent hybridization conditions, other related HPV sequences may be present in these cell lines. Analysis of these cell lines with additional HPV DNA types as they are discovered or analysis of these cell lines under nonstringent hybridization conditions will be necessary.

The regular association of HPV-16 and HPV-18 DNA sequences in biopsies of cervical tumors,[4,7] the presence of HPV DNA sequences in six of eight cervical carcinoma lines, and the demonstration of viral transcription in five of six of these lines argues for an active role of these papillomaviruses in the etiology of these tumors. The papillomaviruses, however, cause persistent and latent infections of tissues; and, at this point, one cannot rule out the possibility that the presence of these viral sequences is merely a consequence of such an infection. A mechanism by which this group of specific HPVs may induce malignant progression will have to be elucidated for the establishment of an etiologic role for the HPVs in human cervical carcinoma. It is certainly possible that viral gene products encoded by these HPVs and expressed in these cell lines may play a role in the establishment and maintenance of these tumors. Transforming genes have been mapped in another well-studied papillomavirus, the bovine papillomavirus.[26-28] Also, integration of the HPV sequences could result in the perturbation of host functions which result in the tumor progression. Studies to evaluate these possibilities are currently under way in our laboratory.

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